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TITLE: Understanding the Function of EZH2 as a Determinant of Breast Cancer Invasion and Metastasis

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14. ABSTRACT

EZH2 exerts oncogenic functions in breast cancer, where its overexpression is associated with metastasis. While it reportedly acts a transcriptional repressor, EZH2 may exhibit context-dependent activating functions. Despite associations with worse outcome and metastasis in breast cancer, a functional role for EZH2 in breast cancer metastasis in vivo has not been demonstrated. Also, whether EZH2 regulates cancer cell phenotype and motility are unknown. We discovered that EZH2 knockdown induces a phenotypic reprogramming from mesenchymal to epithelial, and reduces motility and invasion in breast cancer cells. In vivo, EZH2 knockdown decreased spontaneous metastasis to the lungs in mice. We found a role for EZH2 in inducing the p38 signaling pathway, an important regulator of breast cancer invasion and metastasis. In breast cancer cells, EZH2 bound to phosphorylated p38 (p-p38) in association with other core members of the Polycomb Repressive Complex 2, and EZH2 overexpression led to increased levels of p-p38 and of activated, downstream pathway proteins. The effect on p-p38 was confirmed in vivo as it correlated with decreased spontaneous metastasis. In clinical specimens of matched primary and invasive breast carcinomas, EZH2 expression was up-regulated in 100% of metastases, and EZH2 and p-p38 were co-expressed in 63% of cases. These findings reveal a new mechanism by which EZH2 functions in breast cancer.

15. SUBJECT TERMS
EZH2, Breast Cancer, Invasion, Metastasis, p38

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Introduction

This is the third and final report for a project that aims to understand the function of EZH2 as a determinant of breast cancer invasion and metastasis. EZH2 (Enhancer of Zeste Homolog 2), a Polycomb group protein presumed to function in controlling the transcriptional memory of a cell [1, 2], is up-regulated during progression from ductal carcinoma in situ, the precursor of invasive carcinoma, to invasive carcinoma and distant metastasis [3]. Furthermore, EZH2 protein is over-expressed in 55% of invasive breast carcinomas, and is significantly associated with poorly differentiated tumors [3-5]. Our laboratory has previously shown that EZH2 is a powerful independent prognostic biomarker in breast cancer, providing outcome information above and beyond conventional prognosticators used in the clinical setting [3]. We have also demonstrated by Kaplan-Meier analysis that tumors with high EZH2 expression had a worse disease free and overall survival than tumors with low EZH2 expression. I have hypothesized that EZH2 over-expression may give rise to breast cancers that are on a highly aggressive path from the outset and may drive metastasis. Specifically, we aimed to demonstrate with this award the functional and mechanistic role of EZH2 in invasion and metastasis through both *in vitro* and *in vivo* assays. As illustrated in this report, novel and exciting discoveries during the course of this project have led me to pursue some avenues of my proposal much more vigorously than others and to make minor alterations to the planned experiments within the approved statement of work in order to address these novel discoveries better. For example, we will demonstrate that the p38 mitogen activated kinase (MAPK) pathway is being regulated by EZH2 protein levels (See Aims 2 & 3); these data have led to a shift in the proposed experimental priorities from the Wnt signaling pathway to the p38 MAPK signaling pathway. Although the data has indicated a different signaling pathway of interest, the proposed experiments have essentially remained the same with only the target protein of interest being altered.

Body

Below are brief descriptions of key accomplishments according to the approved statement of work:

Aim 1: To elucidate the role of EZH2 in triggering invasion and metastasis of breast cancer both in vitro and in vivo.

- **Task 1**: To investigate the role of EZH2 on invasion through *in vitro* modified Boyden chamber invasion assays using breast cancer cell lines. (Year 1)
- **Task 2**: To investigate the role of EZH2 on metastasis through the *in vivo* pulmonary metastasis model. (Year 2)

Note: The data presented here supported by the DOD BCRP has been recently published in *Breast Cancer Research and Treatment* in March 2013 under the title "EZH2 inhibition decreases p38 signaling and suppresses breast cancer motility and metastasis" (DOI: 10.1007/s10549-013-2498-x). All references to figures and methods in the following section should be directed to this publication, which is included in full in Appendix 1.

Task 1

We investigated the role of EZH2 on invasion using a modified Boyden chamber assay. This assay determines the ability of a cell to invade through an extracellular matrix by measuring the number of cells invading through a growth factor-reduced Matrigel™-coated transwell chamber over 24 hours [6]. Guided by our previous published data showing that EZH2 overexpression in invasive carcinomas is significantly associated with negative estrogen receptor (ER) status [3], we concentrated our studies on the ER negative (ER^-) breast cancer cell lines MDA-MB-23 and SUM149, both of which are invasive,

tumorigenic in vivo and express high levels of EZH2 protein in comparison to non-tumorigenic breast cell lines [7]. To determine if EZH2 has a role in invasion in these cells, we utilized two independent and complementary methods to downregulate EZH2 protein levels in breast cancer cells: stable expression of short-hairpin RNA interference targeting EZH2 (shEZH2) in lentivirus and pharmacologic inhibition using 3-Deazaneplanocin A (DZNeP), a histone methyltransferase inhibitor which disrupts PRC2 (Fig. 1a & Supplementary Fig. 1a). Knockdown of EZH2 with shRNA or DZNeP significantly reduced invasion in MDA-MB-231 and SUM149 cells when compared to corresponding controls (Fig. 1c, Supplementary Fig. 1c). While conducting the invasion assays, we observed that cells with knockdown of EZH2 displayed different cell morphology when grown in tissue culture. Indeed, EZH2 knockdown through either shRNA or DZNeP treatment was sufficient to induce a morphologic and a molecular mesenchymal-to-epithelial transition (MET) of SUM149 and MDA-MB-231 cells when compared to scrambled shRNA or untreated controls, respectively (Fig. 1a-b & Supplementary Fig. 1a-b). The observed morphological change was associated with a protein expression profile characteristic of MET: increased expression of the epithelial markers Cytokeratin-18 and E-cadherin and decreased expression of the mesenchymal markers Vimentin and Snail1 (Fig. 1a & Supplementary Fig. 1a).

A characteristic of rapidly progressive breast cancer cells is their ability to migrate through normal tissues and invade the surrounding stroma. To test the hypothesis that EZH2 down-regulation in breast cancer cells decreases cell motility, we performed random motion motility assays on MDA-MB-231 cells with EZH2 down-regulation. Live cell imaging was utilized to track cell motility over 24 hour periods with images taken every 10 minutes. Briefly, cells were cultured on collagen-coated chambered coverslips at a low density and imaged using the DeltaVision Imaging System at the University of Michigan Microscopy and Image analysis Laboratory. Using MTrackJ software from ImageJ, we were able to track individual cells over the time period and determine each cell's average velocity (μm/min). EZH2 downregulation by shRNA or DZNeP in MDA-MB-231 cells significantly decreased the average cell velocity when compared to controls (Fig. 2a-b). Furthermore, rescue of EZH2 expression using a myc-tagged EZH2 adenovirus partially reversed the decreased motility induced by EZH2 knockdown in MDA-MB-231 cells (Fig. 2c). Collectively, these experiments show that EZH2 downregulation promotes a MET and reduces the motility and invasiveness of breast cancer cells. Collectively, these data demonstrate that EZH2 downregulation decreases invasion and motility and promotes MET in breast cancer cells *in vitro*.

Task 2

As outlined in the statement of work, we have completed analysis on an *in vivo* metastasis model. Upon further research and consultation into *in vivo* metastasis techniques, we determined that a spontaneous metastasis model would be preferable to the originally proposed pulmonary metastasis model. Therefore, we have conducted analyses of spontaneous lung metastases derived from NOD-SCID mouse xenografts using MDA-MB-231 vector and shEZH2 cells ($2x10^6$ cells injected into the mammary fat pad). Mice were monitored for tumor growth and once primary tumors reached 2 cm^3 in volume, mice were humanely sacrificed and lung tissues, in addition to primary tumors, were collected. The data demonstrating that EZH2 knockdown inhibited primary tumor growth *in vivo* has been previously reported by our laboratory [7]; however, the evaluation of the metastases from these mice had not yet been completed. Compared to the scrambled shRNA control, EZH2 knockdown reduced the ability of MDA-MB-231 cells to form spontaneous lung metastasis when injected into the mammary fat pads of NOD/SCID mice (Fig. 4a-b). Histologic analysis of lung tissues collected when primary tumors reached 2 cm^3, revealed that 8 of 10 (80 %) MDA-MB-231/control mice developed metastases compared with 6 of 10 (60 %) of MDA-MB-231/shEZH2 mice. Although no significant difference was

observed in the number of mice developing lung metastases between conditions, the metastatic burden as determined by the number of lung metastases per mouse was significantly reduced in shEZH2 mice in comparison to control mice (Fig. 4b). In addition, the metastases formed by shEZH2 cells were smaller than controls; the average sizes of the largest lung metastasis per mouse were 304 and 737 μm from shEZH2 and control mice, respectively. Consistent with our functional *in vitro* findings, pathological analyses revealed a change in the invasive pattern of breast cancer cells at the metastatic site. The metastases formed by control cells exhibited irregular and infiltrative borders, and encased pre-existing normal structures such as bronchioles and blood vessels (Fig. 4a). In contrast, metastases formed by shEZH2 cells were smaller and circumscribed, with round borders and minimal parenchymal infiltration (Fig. 4a). Consistent with the *in vitro* data and the histopathologic findings, metastases formed by shEZH2 cells exhibited increased Cytokeratin-18 and decreased Snail1 proteins compared to metastases formed by the controls, as demonstrated by double immunohistochemical analyses (Fig. 4a); this effect was also observed in the primary xenografts (Supplementary Fig. 4a). EZH2 knockdown also significantly reduced cell proliferation in the metastases as measured by Ki67 immunohistochemistry compared to controls (Supplementary Fig. 4b).

Aim 2: To investigate the mechanism of EZH2 in regulating key components of the canonical Wnt signaling pathway in breast cancer.

- **Task 3**: Determine if EZH2 over-expression directly leads to an increase in β-catenin mRNA levels and/or binds the *β-catenin* promoter. (Year 1)
- **Task 4**: Investigate if EZH2 over-expression results in changes in mRNA and/or protein levels of other key components of the Wnt signaling pathway using cells described in Task 3. (Year 1)

Tasks 3 and 4

Our compelling results from Aim 1, tasks 1 and 2, showing that EZH2 is a major regulator of invasion and motility in breast cancer, led us to hypothesize that EZH2 may regulate genes involved in the metastatic process. Unfortunately, we did not find EZH2 over-expression to affect the mRNA or protein levels of β-catenin or of other key components of the Wnt signaling pathway. However, the p38 MAPK has emerged as an important regulator of cell migration and metastasis in breast cancer models [8-11]. Whether EZH2 influences the levels and function of p38 in human breast cancer is unknown. We found that EZH2 downregulation with shRNA or DZNeP reduced phosphorylated p38 (p-p38) protein and the phosphorylation of downstream targets MAPKAPK-2 (MK2) and heat shock protein 27 (HSP27) in breast cancer cell lines when compared to controls (Fig. 3a & Supplementary Fig. 2a). No significant effect on the levels of total p38 was observed by EZH2 knockdown. Conversely, adenoviral overexpression of myc-tagged EZH2 in non-tumorigenic MCF10A breast cells and in MCF7 breast cancer cells, two cell lines normally exhibiting low EZH2 protein expression, consistently led to up-regulation of p-p38 protein levels when compared to controls (Supplementary Fig. 2b). In addition, supporting our *in vitro* observations and mechanistic studies, shEZH2 lung metastases from MDA-MB-231 xenografts had decreased p-p38 levels when compared to controls (Fig. 4c).

To further define the mechanistic link between EZH2 and p38, we tested whether p-p38 regulates the levels of EZH2 and other core components of PRC2. Treatment with SB203580 or SB202190, which inhibit the ability of activated p-p38 to phosphorylate downstream targets, such as HSP27, had no effect on EZH2, SUZ12, EED, or H3K27me3 protein levels in MDA-MB-231 cells. These data indicate that EZH2 levels in breast cancer cells are not affected by p38 pathway activity (Fig. 3b).

We next investigated whether EZH2 regulates a specific p38 isoform by testing the effect of EZH2 knockdown on the expression of phosphorylated p38α, p38β, p38γ, and p38δ. As phospho-specific isoform antibodies are not available, total p-p38 was immunoprecipitated from whole cell extracts of MDA-MB-231 and SUM149 cells expressing scrambled or EZH2-targeted shRNA followed by Western blot analysis for the four isoforms. EZH2 knockdown decreased the phosphorylated levels of all isoforms when compared to controls, while total p38 isoform protein levels remained unaffected (Fig. 3c & Supplementary Fig. 3a). Further supporting a non-transcriptional role for EZH2 in the regulation of p-p38, quantitative real-time RT-PCR showed that neither knockdown nor overexpression of EZH2 affected the mRNA levels of the p38 isoforms when compared to controls (Supplementary Fig. 3b). Collectively, these results show that EZH2 regulates the phosphorylated levels of all p38 isoforms in breast cancer cells and suggest either an indirect transcriptional or a post-transcriptional regulatory mechanism.

To further understand the mechanism by which EZH2 regulates p-p38, we tested the hypothesis that EZH2 may bind to p-p38 in breast cancer cells. IP and Western blot analyses revealed that endogenous EZH2 protein interacts with p-p38 in SUM149 and MDA-MB-231 breast cancer cells; and that the binding is reduced by EZH2 knockdown, thereby supporting the specificity of the interaction (Fig. 3d, e). Furthermore, reciprocal IP experiments demonstrated that EZH2 protein binds to p38/p-p38 protein in association with EED and SUZ12 (Fig. 3e). As PRC2 functions in protein methylation, we hypothesized that PRC2 may methylate p38. Even though all p-p38 isoforms were affected by EZH2 protein expression, we chose to analyze p38α because it is the most abundant and ubiquitously expressed [12]. An *in vitro* methylation assay shows that addition of PRC2 leads to p38α protein methylation (Supplementary Fig. 3c). Collectively, these data demonstrate that EZH2 binds to p-p38 in association with PRC2, and show that PRC2 can methylate p38α *in vitro*, which paves the way for future mechanistic investigations.

Aim 3: To determine the clinical utility of EZH2 and β-catenin detection, amongst other key components of Wnt signaling, in low stage (Stages I and II) invasive breast carcinomas as a predictor of metastasis.

- **Task 5**: To test the prognostic utility of EZH2 and β-catenin in patients' tissues and aim to develop a clinical test to identify patients with highly aggressive tumors at an early anatomic stage (Years 2-3)

The relevance of these novel findings to human breast cancer was validated by examining the expression of EZH2 and p-p38 proteins in a unique cohort of primary invasive carcinomas and their matched metastases from 16 patients, arrayed in tissue microarrays (TMA) [13, 14]. Each tumor, whether primary or metastatic, was represented in the TMA by 5 separate cores to allow for thorough analysis of staining of proteins of interest. When present, EZH2 and p-p38 predominantly localized to the nuclei of breast cancer cells (Fig. 5a-b). EZH2 and p-p38 were scored as exhibiting low or high expression according to a previously validated schema [3, 11]. We found that EZH2 was significantly up-regulated in all metastases when compared to primary carcinomas, and that EZH2 and p-p38 were co-expressed in 63 % of the metastases (Fig. 5c). The complete clinical and pathological information on these tumors is shown in Supplementary Table 1. This *in vivo* data from human breast carcinomas further emphasizes the *in vitro* and mouse *in vivo* data demonstrating that EZH2 plays a role in breast cancer invasion and metastasis, possibly through the p38 MAPK signaling pathway.

Key Research Accomplishments

- EZH2 knockdown in breast cancer cell lines induces a mesenchymal-to-epithelial transition and decreases the ability of breast cancer cells to invade and migrate.
- EZH2 knockdown is sufficient to reduce distant metastasis *in vivo.*
- EZH2 regulates the levels of phosphorylated p38 protein and its signaling pathway *in vitro* and *in vivo*.
- EZH2 protein binds with phosphorylated p38 protein in association with other PRC2 members.
- Human breast cancer metastases exhibit high EZH2 and p-p38 protein expression as determined in a tissue microarray containing 16 matched primary and metastatic breast tumors from the same patients.

Reportable Outcomes

(March 2010 – Present)

Manuscripts/Publications

Gonzalez ME, **Moore HM**, Li X, Toy KA, Kidwell K, Kleer CG. EZH2 expands the stem cell population in benign and tumorigenic breast cells through regulation of Notch1 signaling. *Manuscript in preparation for PNAS*.

Moore HM, Gonzalez ME, Toy KA, Cimino-Mathews A, Argani P, Kleer CG. 2013. EZH2 inhibition decreases p38 signaling and suppresses breast cancer motility and metastasis. *Breast Cancer Research and Treatment, In Press*.

Gonzalez ME, DuPrie ML, **Krueger H**, Merajver SD, Ventura AC, Toy KA, Kleer CG. 2011. Histone methyltransferase EZH2 induces Akt-dependent genomic instability and BRCA1 inhibition in breast cancer. *Cancer Research*, 71(6):2360-70.

Published Abstracts

(Completed Poster Presentations)

Moore H, Gonzalez ME, Daniels CN, Li X, Toy K and Kleer CG. Understanding the Function of EZH2 as a Determinant of Breast Cancer Invasion and Metastasis. 32nd Annual Cellular and Molecular Biology Program Symposium, University of Michigan, Ann Arbor, MI, September 2012.

Moore H, Gonzalez ME, Daniels CN, Li X, Toy K and Kleer CG. Understanding the Function of EZH2 as a Determinant of Breast Cancer Invasion and Metastasis. 103rd Annual Meeting American Association for Cancer Research, Chicago, IL, March 2012.

Moore H, Gonzalez ME, Daniels CN, Li X, Toy K and Kleer CG. Understanding the Function of EZH2 as a Determinant of Breast Cancer Invasion and Metastasis. Rackham Centennial Symposium, University of Michigan, Ann Arbor, MI, February 2012.

Moore H, Gonzalez ME, Li X, Toy K and Kleer CG. Understanding the Function of EZH2 as a Determinant of Breast Cancer Invasion and Metastasis. 23nd Annual Cancer Center Research Fall Symposium, University of Michigan, Ann Arbor, MI, November 2011.

Krueger H, Gonzalez ME, Li X, Toy K and Kleer CG. EZH2 Regulates the Self Renewal of Mammary Stem Cells Through Notch1 Signaling. 31st Annual Cellular and Molecular Biology Program Symposium, University of Michigan, Ann Arbor, MI, September 2011.

Krueger H, Gonzalez ME, Li X, Toy K and Kleer CG. EZH2 Regulates the Self Renewal of Mammary Stem Cells Through Notch1 Signaling. Department of Defense Breast Cancer Research Program 6th Era of Hope Conference, Orlando, FL, August 2011.

Gonzalez ME, Li X, **Krueger H**, Toy K and Kleer CG. EZH2 Promotes Expansion of Breast Tumor Initiating Cells through Activation of Notch1-Jagged Signaling. 102nd Annual Meeting American Association for Cancer Research, Orlando, FL, April 2011.

Krueger H, Gonzalez ME, Li X, Toy K and Kleer CG. The Role of EZH2 in the Self Renewal and Carcinogenesis of Mammary Stem Cells. 30th Annual Cellular and Molecular Biology Program Symposium, University of Michigan, Ann Arbor, MI, September 2010.

Gonzalez ME, DuPrie M, Toy K, Ventura A, **Krueger H**, Li X and Kleer CG. EZH2 Regulates BRCA1 Transient Nuclear Export during the Cell Cycle and Induces Tetraploidy of Breast Cells. 101st Annual Meeting American Association for Cancer Research, Washington, D.C., April 2010.

Presentations

"The Role of EZH2 in Breast Cancer Progression and Metastasis" Cellular and Molecular Biology Dissertation Seminar, University of Michigan, Ann Arbor, MI, April 25, 2013.

"Understanding the Role of EZH2 in Breast Cancer Invasion and Metastasis" 3rd Annual Cellular and Molecular Biology Retreat, Kellogg Biological Station, Battle Creek, MI, May 19, 2012.

"The Role of EZH2 in Breast Cancer Development and Metastasis" Cellular and Molecular Biology Seminar Series, University of Michigan, Ann Arbor, MI, February 7, 2011.

Degree Obtained

A doctoral dissertation entitled "The Role of EZH2 in Breast Cancer Progression and Metastasis" has been completed. An oral defense is scheduled for April 25, 2013. Ph.D. degree will be conferred August 16, 2013.

Funding
(Based on research supported by this award):

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Employment Applications

Agency Name/Job Title: Federal Bureau of Investigation/Intelligence Analyst Register, (External)
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Announcement #: FO-2012-0102
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Agency Name/Job Title: Patent and Trademark Office/Patent Examiner - Biological Science
Series & Grade: GS-1224-09
Announcement #: CP-2012-0106
Status: Phone Interview Conducted – Application Not Referred to Selecting Official, Applied 2/20/2012

Agency Name/Job Title: Food and Drug Administration/Interdisciplinary Scientist
Series & Grade: GS-0401/1310-13
Announcement #: PH-NM-12-603871
Status: Eligible – Application Not Referred to Selecting Official, Applied 2/17/2012

Agency Name/Job Title: Multi-Agency/2012 Presidential Management Fellows Program
Series & Grade: GS-0300-09
Announcement #: 519543
Status: Eligible – Application Not Referred to Selecting Official, Applied 9/25/2011

Conclusions

EZH2 is a Polycomb group protein that exerts oncogenic functions in breast cancer, where its overexpression is associated with metastatic disease. While it reportedly acts a transcriptional repressor through trimethylation of histone H3 at lysine 27, EZH2 may exhibit context-dependent activating functions. Despite associations with worse outcome and metastasis in breast cancer, a functional role of EZH2 in breast cancer metastasis *in vivo* has not been demonstrated. Furthermore, whether EZH2 regulates cancer cell phenotype and motility are unknown. In this study supported by the Department of Defense Breast Cancer Research Program, we discovered that knockdown of EZH2 induces a phenotypic reprogramming from mesenchymal to epithelial, reduces motility, and blocks invasion in breast cancer cell lines. *In vivo*, EZH2 downregulation in MDA-MB-231 cells decreased spontaneous metastasis to the lungs. We uncovered an unexpected role of EZH2 in inducing the p38 mitogen-activated protein kinase signaling pathway, an important regulator of breast cancer invasion and metastasis. In breast cancer cells, EZH2 bound to phosphorylated p38 (p-p38) in association with other core members of the Polycomb repressive complex 2, EED, and SUZ12, and EZH2 overexpression led to increased levels of p-p38 and of activated, downstream pathway proteins. The effect on p-p38 was confirmed *in vivo*, where it correlated with decreased spontaneous metastasis. In clinical specimens of matched primary and invasive breast carcinomas, we found that EZH2 expression was upregulated in 100 % of the metastases, and that EZH2 and p-p38 were coexpressed in 63 % of cases, consistent with the functional results. Together our findings reveal a new mechanism by which EZH2 functions in breast cancer, and provide direct evidence that EZH2 inhibition reduces breast cancer metastasis *in vivo* indicating that targeting EZH2 may have therapeutic implications for human patients.

Training Accomplishments

In addition to my research outlined above, I have received excellent training in breast cancer research through constant interaction and guidance with my faculty mentor Dr. Celina Kleer, who is a breast cancer pathologist with the University of Michigan. In addition, I have received guidance from my thesis committee members, my laboratory colleagues, and the faculty and fellow peers in the Cellular and Molecular Biology Program. During my funding period, I have been able to present my work at several conferences and symposiums (see reportable outcomes for listings) receiving critical feedback that I have been able to implement into my studies and conclusions. I have also attended the biweekly Breast Cancer Educational Forum seminars presented at the University of Michigan by local and visiting researchers and clinicians gaining incredible insight beyond the bench. Moreover, I have had the priceless experience of seeing a manuscript all the way through the peer review and publication process this past year. Most importantly, the funding provided by the Department of Defense Breast Cancer Research Program has allowed me to complete my thesis dissertation and I will earn my doctoral degree by the end of the current academic term. I am enthusiastic to continue in the field of breast cancer research after the completion of this funding award and my degree.

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Breast Cancer Res Treat
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PRECLINICAL STUDY

EZH2 inhibition decreases p38 signaling and suppresses breast cancer motility and metastasis

Heather M. Moore · Maria E. Gonzalez · Kathy A. Toy · Ashley Cimino-Mathews · Pedram Argani · Celina G. Kleer

Received: 8 February 2013 / Accepted: 21 March 2013

Abstract EZH2 is a Polycomb group protein that exerts oncogenic functions in breast cancer, where its overexpression is associated with metastatic disease. While it reportedly acts a transcriptional repressor through trimethylation of histone H3 at lysine 27, EZH2 may exhibit context-dependent activating functions. Despite associations with worse outcome and metastasis in breast cancer, a functional role of EZH2 in breast cancer metastasis in vivo has not been demonstrated. Furthermore, whether EZH2 regulates cancer cell phenotype and motility are unknown. In this study, we discovered that knockdown of EZH2 induces a phenotypic reprogramming from mesenchymal to epithelial, reduces motility, and blocks invasion in breast cancer cell lines. In vivo, EZH2 downregulation in MDA-MB-231 cells decreases spontaneous metastasis to the lungs. We uncover an unexpected role of EZH2 in inducing the p38 mitogen-activated protein kinase signaling pathway, an important regulator of breast cancer invasion and metastasis. In breast cancer cells, EZH2 binds to phosphorylated p38 (p-p38) in association with other core members of the Polycomb repressive complex 2, EED, and SUZ12, and EZH2 overexpression leads to increased levels of p-p38 and of activated, downstream pathway proteins. The effect on p-p38 was confirmed in vivo, where it correlated with decreased spontaneous metastasis. In clinical specimens of matched primary and invasive breast carcinomas, we found that EZH2 expression was upregulated in 100 % of the metastases, and that EZH2 and p-p38 were coexpressed in 63 % of cases, consistent with the functional results. Together our findings reveal a new mechanism by which EZH2 functions in breast cancer, and provide direct evidence that EZH2 inhibition reduces breast cancer metastasis in vivo.

Keywords Breast cancer · EZH2 · p38 · Cell motility · Metastasis · Snail

Abbreviation

EMT	Epithelial-to-mesenchymal transition
EZH2	Enhancer of zeste homolog 2
MET	Mesenchymal-to-epithelial transition
p-p38 MAPK	Phosphorylated p38 mitogen-activated protein kinase
PRC2	Polycomb repressive complex 2

Electronic supplementary material The online version of this article (doi:10.1007/s10549-013-2498-x) contains supplementary material, which is available to authorized users.

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Introduction

Breast cancer is the 2nd most common cause of cancer-related deaths for women in the United States [1]. Despite advances in breast cancer detection and treatment strategies, metastatic breast cancer is essentially incurable and the 5-year-survival rate for women diagnosed with distant metastatic disease is only 23 % [2]. The degree of breast cancer cell differentiation directly impacts its metastatic ability; the more undifferentiated the primary invasive carcinoma, the greater likelihood to develop metastasis [3]. Thus, it is not surprising that dysregulation of cell type identity and differentiation programs directly impact breast cancer metastasis.

Polycomb group proteins are major regulators of cellular memory that function in multimeric complexes to regulate the expression of specific genes, mainly through transcriptional repression. Enhancer of zeste homolog 2 (EZH2) is the catalytic core member of the Polycomb repressive complex 2 (PRC2), which catalyzes the trimethylation of histone H3 lysine27 (H3K27me3) [4–6]. Although primarily functioning in gene repression, EZH2 has been shown to exhibit gene activating functions, at times through mechanisms independent of its histone methyltransferase activity [7–10]. EZH2 is highly expressed in a wide range of human cancers and has been shown to mediate the expression of target genes involved in tumorigenesis, including cell cycle regulation and proliferation, stem cell maintenance, cell differentiation, and neoplastic cell transformation [11–13]. EZH2 protein is overexpressed in 55 % of invasive breast carcinomas, and is significantly associated with poorly differentiated, estrogen receptor negative (ER^-) tumors [14–17]. We have demonstrated that EZH2 is an independent prognostic biomarker in breast cancer as women with tumors expressing high EZH2 have worse disease free and overall survival than women with tumors expressing low EZH2 [14]. Despite these associations, direct demonstration that EZH2 downregulation decreases breast cancer metastasis is lacking.

The p38 mitogen-activated protein kinase (MAPK) signaling pathway plays a complex and key role in cancer progression by translating extracellular signals into cellular responses through phosphorylation of specific serine and threonine residues of downstream effector proteins, especially transcription factors and protein kinases. Four p38 isoforms have been identified, whose implications in tumorigenesis may depend on cell context and tumor type [19, 20]. Once activated, p38 has been associated with regulation of the epithelial-to-mesenchymal transition (EMT), invasion and motility of cancer cells, all cellular processes that are crucial to metastasis [19, 20]. Recently, elevated p38γ expression was shown to be associated with a lower overall survival of patients with breast cancer [21].

In this study, we demonstrate a previously undescribed function of EZH2: its role in cancer cell motility and cell phenotype. EZH2 knockdown in breast cancer cells induces a mesenchymal-to-epithelial transition (MET), decreases cancer cell motility and the speed of movement. We provide first evidence that EZH2 knockdown in breast cancer cells reduces lung metastasis in vivo. Mechanistically, EZH2 binds to phosphorylated p38 (p-p38) and upregulates p38 downstream signaling, while EZH2 inhibition in breast cancer cells decreases p-p38 binding, expression, and downstream signaling. The relevance of our in vivo and in vitro studies to human breast cancer is highlighted by the finding that human breast cancer distant metastases express high levels of EZH2 and p-p38. Taken together, this study identifies a novel function of EZH2 in controlling p-p38 activity, breast cancer cell motility, and metastasis.

Materials and methods

Cell culture

Breast cancer cell lines MDA-MB-231 and MCF7 and mammary epithelial cell line MCF10A were obtained from the American Type Culture Collection. All cell lines were grown under recommended conditions. The SUM149 breast cancer cell line was obtained from the S. Ethier Laboratory (Karmanos Cancer Institute) and cultured as previously reported [22].

Enhancer of zeste homolog 2 knockdown using stable short-hairpin interfering RNA in lentivirus was completed as previously reported [17]. Cells were transduced and selected for antibiotic resistance with puromycin (Sigma-Aldrich, #P9620). EZH2 knockdown was also achieved using 3-Deazaneplanocin A (Cayman Chemical, #13828) at 1 μM for 5 days treating every other day. As previously reported, transient EZH2 overexpression was achieved through infection with an EZH2-encoding, myc-tagged pCMV for 48 h [18, 23, 24]. The p-p38 inhibitors, SB203580 (Cell Signaling, #5633) or SB202190 (Abcam, #120638) were used at 10 or 20 μM for 48 h.

Western blotting and immunoprecipitations

Cells were lysed in RIPA lysis buffer with protease and phosphatase inhibitors (Thermo Scientific, #89900, #78410 & #78420) and Western blot analyses were carried out using 50 μg of whole cell extract. Samples were separated by SDS-PAGE gels and transferred onto PVDF membranes; membranes were blocked and incubated with primary antibodies in 3 % BSA (Sigma-Aldrich, #A3059) in

TBS-T (Bio-Rad, #161-0372, with 0.05 % Tween20) at 4 °C overnight. Protein signals were visualized via chemiluminescence as described by manufacturer (Thermo Scientific, #32106). β-Actin-HRP (Santa Cruz, #47778) was used to confirm equal loading. Cell Signaling antibodies: rabbit monoclonals EZH2 (#5246), E-cadherin (#3195), SUZ12 (#3737), p38β (#2339), p38δ (#2308), MAPKAPK-2 (#3042), Snail1 (#3879); rabbit polyclonals p38 (#9212), p38α (#9218), p38γ (#2307), phospho-HSP27 (Ser82, #2401), phospho-MAPKAPK-2 (#3007); mouse monoclonals Snail1 (#3895) and HSP27 (#2402). Abcam antibodies: rabbit monoclonal Cytokeratin-18 (#32118), rabbit polyclonal EED (#4469), and mouse monoclonal trimethyl-Histone H3 (#6002). Additionally: rabbit polyclonal ACTIVE®-p38 MAPK (pTGpY, Promega, #V1211) and rabbit monoclonal Vimentin (Epitomics, #2707-1).

Immunoprecipitations (IPs) were conducted following protocol instructions (Sigma-Aldrich, #IP50). Protein was extracted from 70 % confluent cells, and protein extracts were precleared with Protein G agarose for 3 h and incubated with antibody (normal mouse IgG [Santa Cruz, #2025], p38 [Novus Biologicals, #NBP1-97545], EED [abcam, #4469], EZH2, phospho-p38, or SUZ12 [Cell Signaling, #5246, #9216, #3737, respectively]) overnight at 4 °C. Next day, protein–antibody complexes were captured with Protein G agarose beads for 2 h, washed in stringent conditions and eluted. Inputs and IPs were separated as by described western blot protocol. Immunoprecipitated EED was detected using Clean-Blot™ IP HRP (Thermo Scientific, #21230) to avoid interference from denatured IgG.

Invasion and motility assays

In vitro invasion was performed using Matrigel Invasion Chambers (BD Biosciences, #354480) according to the manufacturer's instructions, in triplicate. Invasive cells on lower sides of chambers were crystal violet stained, air-dried, and photographed. They were quantified using ImageJ to count colored pixels, or for colorimetric assays, inserts were treated with 10 % acetic acid to remove dye and absorbance was measured at 560 nm.

Random motion cell motility assays were carried out as previously described [21]. In brief, cells were plated on collagen-coated chambered coverslips at low density attaching overnight. Next day, cells were imaged every 10 min at 37 °C for 24 h using the DeltaVision RT Live Cell Imaging System (Applied Precision, GE Healthcare) equipped with a UPlanAo 20X/0.7 NA lens at the University of Michigan Microscopy and Image analysis Laboratory. DIC images were acquired using SoftWoRx 3.5.1 software, and cell movements were quantified using MTrackJ/ImageJ software.

Spontaneous metastasis assay/human breast tissue and immunohistochemistry

Ten-week-old severe combined immunodeficiency mice (Jackson Laboratories) were used for examining tumorigenicity as previously reported [17]. Additional methods on the spontaneous metastasis assay, including information on tumor staining and staining quantifications, can be found in the Supplementary Methods.

A high-density tissue microarray containing 16 human primary invasive breast carcinomas with matched metastases was employed [25, 26]. Immunohistochemistry on formalin-fixed, paraffin-embedded tissue blocks was performed using anti-EZH2 (Cell Signaling, #5246, 1:150) and anti-phospho-p38 MAPK (Cell Signaling, #9216, 1:3,000). EZH2 and p-p38 expression were evaluated as low or high based on the intensity of staining and percentage of staining cells, following published literature [27]. The complete clinical and pathological information on these tumors is shown in Supplementary Table 1.

Results

EZH2 knockdown induces a mesenchymal-to-epithelial transition and decreases the ability of breast cancer cells to move

EZH2 overexpressing breast carcinomas have aggressive clinical behavior, high frequency of estrogen receptor negative status (ER^-) and are associated with a high propensity to metastasize [14]. However, direct evidence that EZH2 regulates cancer cell phenotype and motility are lacking. To test the hypothesis that EZH2 knockdown may reduce the ability of breast cancer cells to move and invade into the surrounding tissues, we employed breast cancer cell lines MDA-MB-231 and SUM149, both of which are ER^-, invasive, tumorigenic in vivo and express high levels of EZH2 protein in comparison to non-tumorigenic breast cell lines [17]. We utilized two independent and complementary methods to downregulate EZH2 protein levels in breast cancer cells: stable expression of a short-hairpin RNA interference (shRNA) in a lentiviral vector and pharmacologic inhibition using 3-Deazaneplanocin A (DZNeP), a histone methyltransferase inhibitor which disrupts PRC2 (Fig. 1a, Supplementary Fig. 1a).

EZH2 knockdown through either shRNA or DZNeP treatment was sufficient to induce a morphologic and a molecular MET of SUM149 and MDA-MB-231 cells when compared to scrambled shRNA or untreated controls, respectively (Fig. 1a, b, Supplementary Fig. 1a, b). The observed morphological change was associated with a protein expression profile characteristic of MET: increased

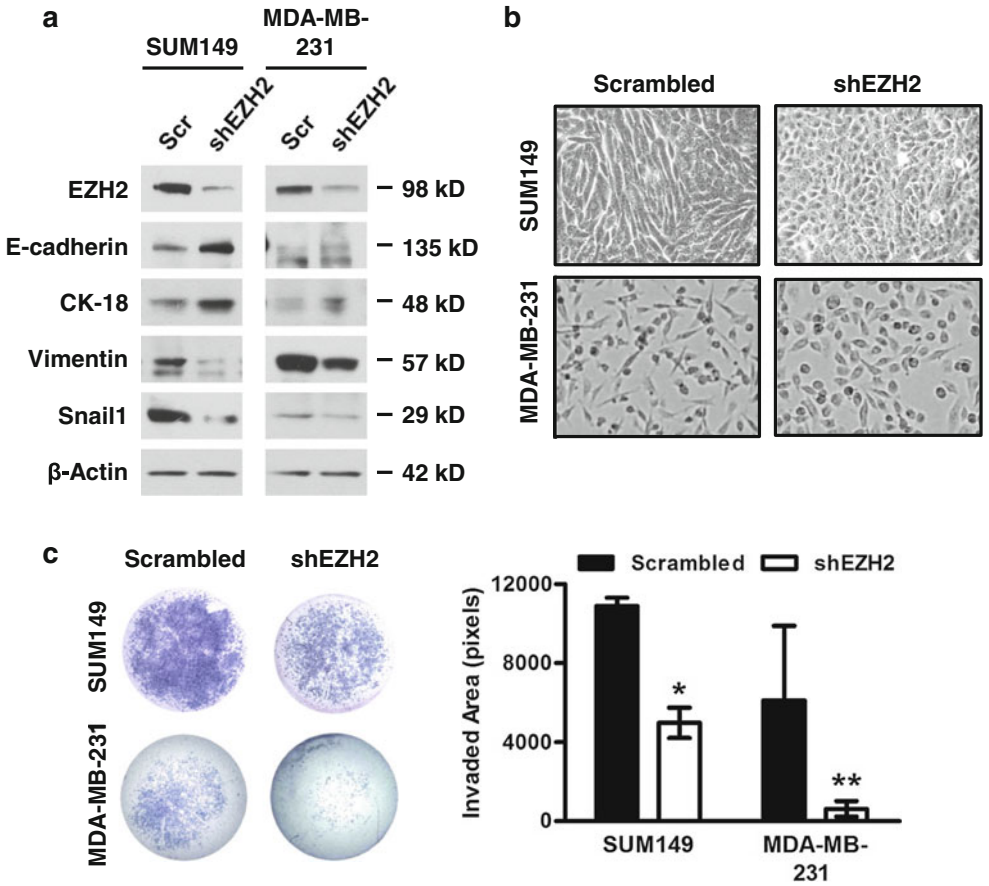


Fig. 1 EZH2 knockdown induces a MET and decreases invasion in breast cancer cells. **a** Immunoblots of SUM149 and MDA-MB-231 breast cancer cells show that downregulation of EZH2 protein with EZH2-targeted shRNA (shEZH2) leads to a protein expression profile indicative of epithelial differentiation compared to scrambled control shRNA (Scr). E-cadherin and Cytokeratin-18 (CK-18) represent epithelial marker proteins, and Vimentin and Snail1 represent mesenchymal marker proteins. **b** Representative phase contrast images show that EZH2 knockdown (KD) in SUM149 and MDA-MB-231 cells leads to a morphological change from mesenchymal-like to epithelial when compared to controls (×200 magnification). **c** EZH2 KD reduces invasion of SUM149 and MDA-MB-231 cells compared to controls using a reconstituted Boyden basement membrane invasion chamber assay. *Left*, representative images of entire invaded and stained chambers are shown; *right*, mean invaded area ± SD was calculated by quantifying stained image pixels using ImageJ (Student's *t* test, $*p < 0.0002$, $**p = 0.03$)

expression of the epithelial markers Cytokeratin-18 and E-cadherin and decreased expression of the mesenchymal markers Vimentin and Snail1 (Fig. 1a, Supplementary Fig. 1a). Knockdown of EZH2 with shRNA or DZNeP significantly reduced invasion in MDA-MB-231 and SUM149 cells when compared to corresponding controls (Fig. 1c, Supplementary Fig. 1c).

We next investigated the role of EZH2 on cell motility, a critical step in metastasis. Random cell motion was quantified using live cell imaging with time-lapse microscopy [21]. EZH2 downregulation by shRNA or DZNeP in MDA-MB-231 cells significantly decreased the average cell velocity when compared to controls (Fig. 2a, b). Furthermore, rescue of EZH2 expression using a myc-tagged EZH2 adenovirus partially reversed the decreased motility induced by EZH2 knockdown in MDA-MB-231 cells (Fig. 2c). Collectively, these experiments show that EZH2 downregulation promotes a MET and reduces the motility and invasiveness of breast cancer cells.

EZH2 regulates the levels of phosphorylated p38 protein and signaling pathway

The p38 MAPK has emerged as an important regulator of cell migration and metastasis in breast cancer models [20, 21, 27]. Whether EZH2 influences the levels and function of p38 in human breast cancer is unknown. We found that EZH2 downregulation with shRNA or DZNeP reduced p-p38 protein and the phosphorylation of downstream targets MAPKAPK-2 (MK2) and heat shock protein 27 (HSP27) in breast cancer cell lines when compared to controls (Fig. 3a, Supplementary Fig. 2a). No significant effect on the levels of total p38 was observed by EZH2 knockdown. Conversely, adenoviral overexpression of

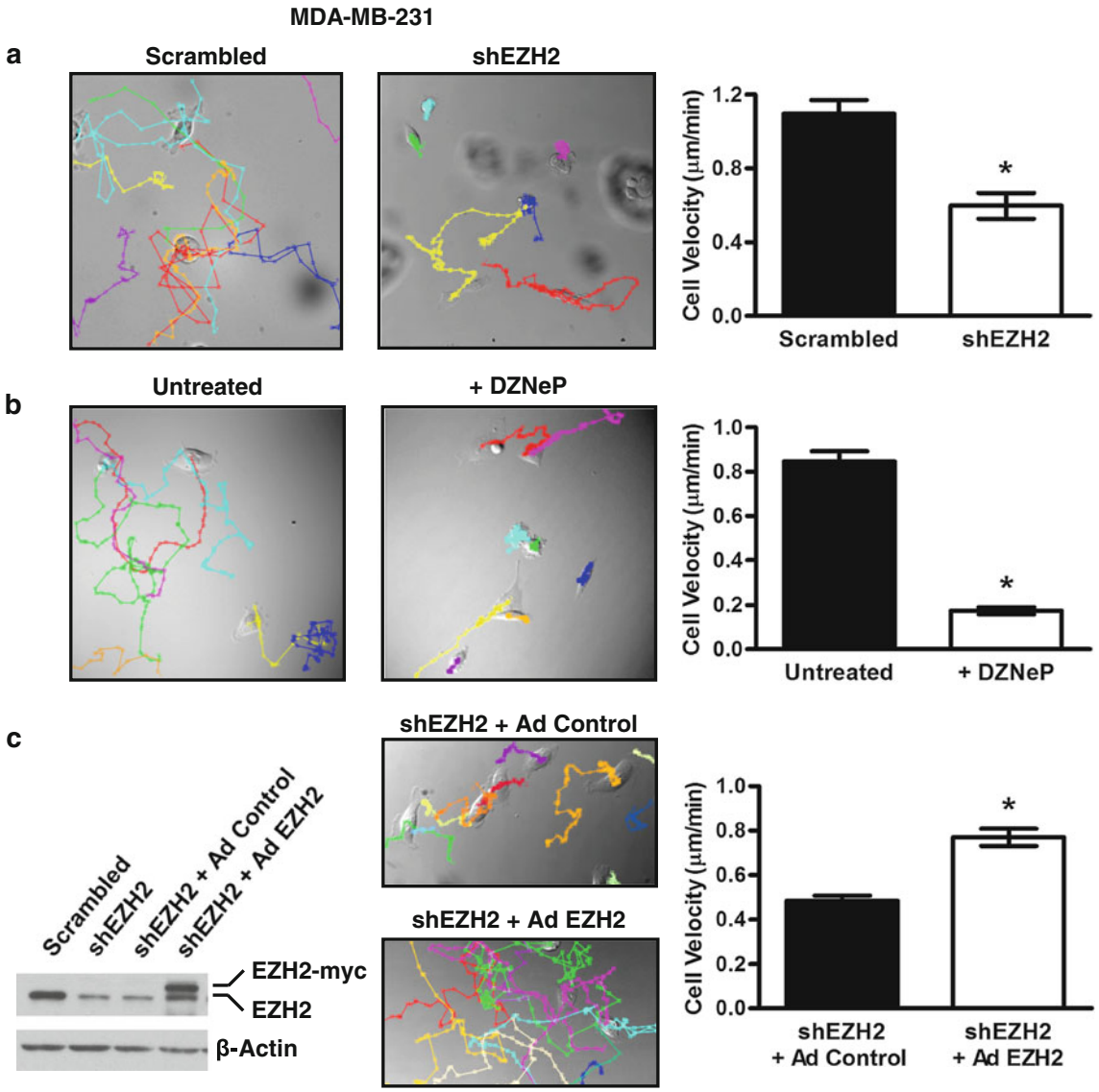


Fig. 2 EZH2 knockdown decreases breast cancer cell motility. **a** and **b** *Left*, representative images displaying MTrackJ individual MDA-MB-231 cell tracks, *colored dots*, and *connecting lines*, from 24 h time-lapse videos of **a** scrambled shRNA control and shEZH2 or **b** untreated and DZNeP treated cells (×200 magnification). Each *dot* represents a 10 min time span and *closely spaced dots* indicate less movement over the elapsed time versus *widely spaced dots*. *Right*, *bar graphs* show that EZH2 KD cells are significantly slower than controls as demonstrated by the average cell velocity ± SEM (Student's *t* test, $*p < 1\times10^{-5}$, $n \geq 25$ cells per condition). **c** Transient rescue of EZH2 expression in MDA-MB-231 EZH2 KD cells using a myc-tagged EZH2-encoding adenovirus reverses the decreased motility of EZH2 KD cells. Representative images displaying cell tracks of shEZH2 cells infected with either control or EZH2 adenovirus [×200 magnification]. The *bar graph* shows that shEZH2 cells with EZH2 adenoviral rescue are significantly faster than control adenoviral infected cells as demonstrated by the average cell velocity ± SEM [Student's *t* test, $*p < 9\times10^{-10}$, $n \geq 90$ cells per condition]

myc-tagged EZH2 in non-tumorigenic MCF10A breast cells and in MCF7 breast cancer cells consistently led to upregulation of p-p38 protein levels when compared to controls (Supplementary Fig. 2b).

To further define the mechanistic link between EZH2 and p38, we tested whether p-p38 regulates the levels of EZH2 and other core components of PRC2. Treatment with SB203580 or SB202190, which inhibit the ability of activated p-p38 to phosphorylate downstream targets, such as HSP27, had no effect on EZH2, SUZ12, EED, or H3K27me3 protein levels in MDA-MB-231 cells. These data indicate that EZH2 levels in breast cancer cells are not affected by p38 pathway activity (Fig. 3b).

We next investigated whether EZH2 regulates a specific p38 isoform by testing the effect of EZH2 knockdown on the expression of phosphorylated p38α, p38β, p38γ, and p38δ. As phospho-specific isoform antibodies are not available, total p-p38 was immunoprecipitated

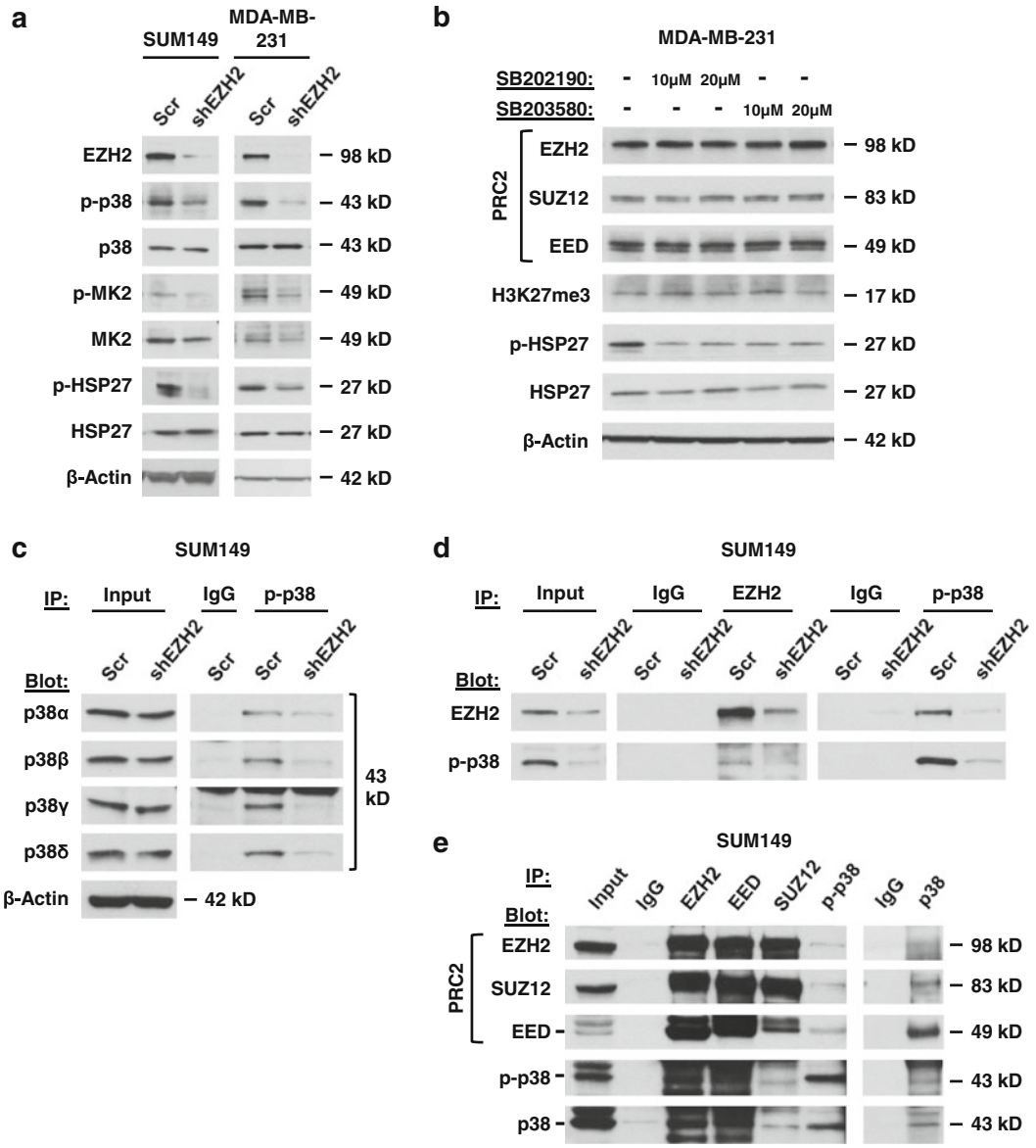


Fig. 3 EZH2 regulates the activation of the p38 MAPK signaling pathway and binds to phosphorylated p38 (p-p38). **a** Immunoblots of SUM149 and MDA-MB-231 breast cancer cells show downregulation of EZH2 protein with EZH2-targeted shRNA (shEZH2) have decreased levels of p-p38 and its activity as demonstrated by the phosphorylation of downstream signaling targets, MK2 and HSP27 when compared to scrambled shRNA control cells (Scr). **b** Immunoblots show that inhibition of p-p38 activity in MDA-MB-231 cells with SB202190 or SB203580 at two different concentrations for 48 h does not affect the levels of EZH2, SUZ12, EED, or H3K27me3. **c** Activated, phosphorylated levels of all four p38 isoforms, but not total isoform protein levels, are decreased in SUM149 shEZH2 cells when compared to scrambled shRNA control cells. Total p-p38 was immunoprecipitated from whole cell extracts followed by Western blot analysis for the four individual isoforms. **d** Co-immunoprecipitations from whole cell extracts of SUM149 shEZH2 and scrambled shRNA control cells show that endogenous EZH2 immunoprecipitates with endogenous p-p38. Extracts were immunoprecipitated with EZH2, p-p38 or control IgG and bound proteins were revealed by Western blot via antibodies against EZH2 and p-p38. **e** Co-immunoprecipitations from whole cell extracts of SUM149 cells show that EZH2 binds p38/p-p38 in association with PRC2 members SUZ12 and EED. Extracts were immunoprecipitated with EZH2, p38, p-p38, EED, SUZ12 or control IgG and bound proteins were revealed by Western blot via antibodies against EZH2, p38, p-p38, EED, and SUZ12

from whole cell extracts of MDA-MB-231 and SUM149 cells expressing scrambled or EZH2-targeted shRNA followed by Western blot analysis for the four isoforms. EZH2 knockdown decreased the phosphorylated levels of all isoforms when compared to controls, while total p38 isoform protein levels remained unaffected (Fig. 3c, Supplementary Fig. 3a). Further supporting a non-transcriptional role for EZH2 in the regulation of p-p38, quantitative real-time RT-PCR showed that neither knockdown nor overexpression of EZH2 affected the mRNA levels of the p38 isoforms when

compared to controls (Supplementary Fig. 3b). Collectively, these results show that EZH2 regulates the phosphorylated levels of all p38 isoforms in breast cancer cells and suggest either an indirect transcriptional or a post-transcriptional regulatory mechanism.

EZH2 protein binds with phosphorylated p38 protein

To further understand the mechanism by which EZH2 regulates p-p38, we tested the hypothesis that EZH2 may bind to p-p38 in breast cancer cells. IP and Western blot analyses revealed that endogenous EZH2 protein interacts with p-p38 in SUM149 and MDA-MB-231 breast cancer cells; and that the binding is reduced by EZH2 knockdown, thereby supporting the specificity of the interaction (Fig. 3d, e). Furthermore, reciprocal IP experiments demonstrated that EZH2 protein binds to p38/p-p38 protein in association with EED and SUZ12 (Fig. 3e). As PRC2 functions in protein methylation, we hypothesized that PRC2 may methylate p38. Even though all p-p38 isoforms were affected by EZH2 protein expression, we chose to analyze p38α because it is the most abundant and ubiquitously expressed [28]. An in vitro methylation assay shows that addition of PRC2 leads to p38α protein methylation (Supplementary Fig. 3c). Collectively, these data demonstrate that EZH2 binds to p-p38 in association with PRC2, and show that PRC2 can methylate p38α in vitro, which paves the way for future mechanistic investigations.

EZH2 knockdown is sufficient to reduce distant metastasis

We have demonstrated that EZH2 knockdown decreases primary breast cancer tumor growth, but whether EZH2 downregulation impacts distant metastasis in breast cancer is unknown [17]. Compared to the scrambled shRNA control, EZH2 knockdown reduced the ability of MDA-MB-231 cells to form spontaneous lung metastasis when injected into the mammary fat pads of NOD/SCID mice (Fig. 4a, b). Histologic analysis of lung tissues collected when primary tumors reached 2 cm^3, revealed that 8 of 10 (80 %) MDA-MB-231/control mice developed metastases compared with 6 of 10 (60 %) of MDA-MB-231/shEZH2 mice. Although no significant difference was observed in the number of mice developing lung metastases between conditions, the metastatic burden as determined by the number of lung metastases per mouse was significantly reduced in shEZH2 mice in comparison to control mice (Fig. 4b). In addition, the metastases formed by shEZH2 cells were smaller than controls; the average sizes of the largest lung metastasis per mouse were 304 and 737 μm from shEZH2 and control mice, respectively.

Consistent with our functional findings, pathological analyses revealed a change in the invasive pattern of breast cancer cells at the metastatic site. The metastases formed by control cells exhibited irregular and infiltrative borders, and encased pre-existing normal structures such as bronchioles and blood vessels (Fig. 4a). In contrast, metastases formed by shEZH2 cells were smaller and circumscribed, with round borders and minimal parenchymal infiltration (Fig. 4a). Consistent with the in vitro data and the histopathologic findings, metastases formed by shEZH2 cells exhibited increased Cytokeratin-18 and decreased Snail1 proteins compared to metastases formed by the controls, as demonstrated by double immunohistochemical analyses (Fig. 4a); this effect was also observed in the primary xenografts (Supplementary Fig. 4a). EZH2 knockdown also significantly reduced cell proliferation in the metastases compared to controls (Supplementary Fig. 4b). Supporting our in vitro observations and mechanistic studies, shEZH2 lung metastases had decreased p-p38 levels when compared to controls (Fig. 4c).

Human breast cancer metastasis exhibit high EZH2 and p-p38 protein expression

The relevance of these novel findings to human breast cancer was validated by examining the expression of EZH2 and p-p38 proteins in a unique cohort of primary invasive carcinomas and their matched metastases from 16 patients, arrayed in tissue microarrays [25, 26]. When present, both proteins predominantly localized to the nuclei of breast cancer cells (Fig. 5a, b). EZH2 and p-p38 were scored as exhibiting low or high expression according to a previously validated schema [14, 27]. We found that EZH2 was significantly upregulated in all metastases when compared to primary carcinomas, and that EZH2 and p-p38 were co-expressed in 63 % of the metastases (Fig. 5c). The complete clinical and pathological information on these tumors is shown in Supplementary Table 1.

Discussion

The data presented here reveal the previously undescribed findings that downregulation of EZH2 leads to MET, decreases motility, and is sufficient to reduce distant metastasis of breast cancer cells in vivo. We uncovered a novel mechanism of EZH2 function by which EZH2 protein binds to p-p38 and leads to upregulated expression of p-p38 protein and its signaling pathway in breast cancer cells.

It has become increasingly evident that cancer cell plasticity influences the biologic behavior of breast cancer by allowing the conversion between epithelial and

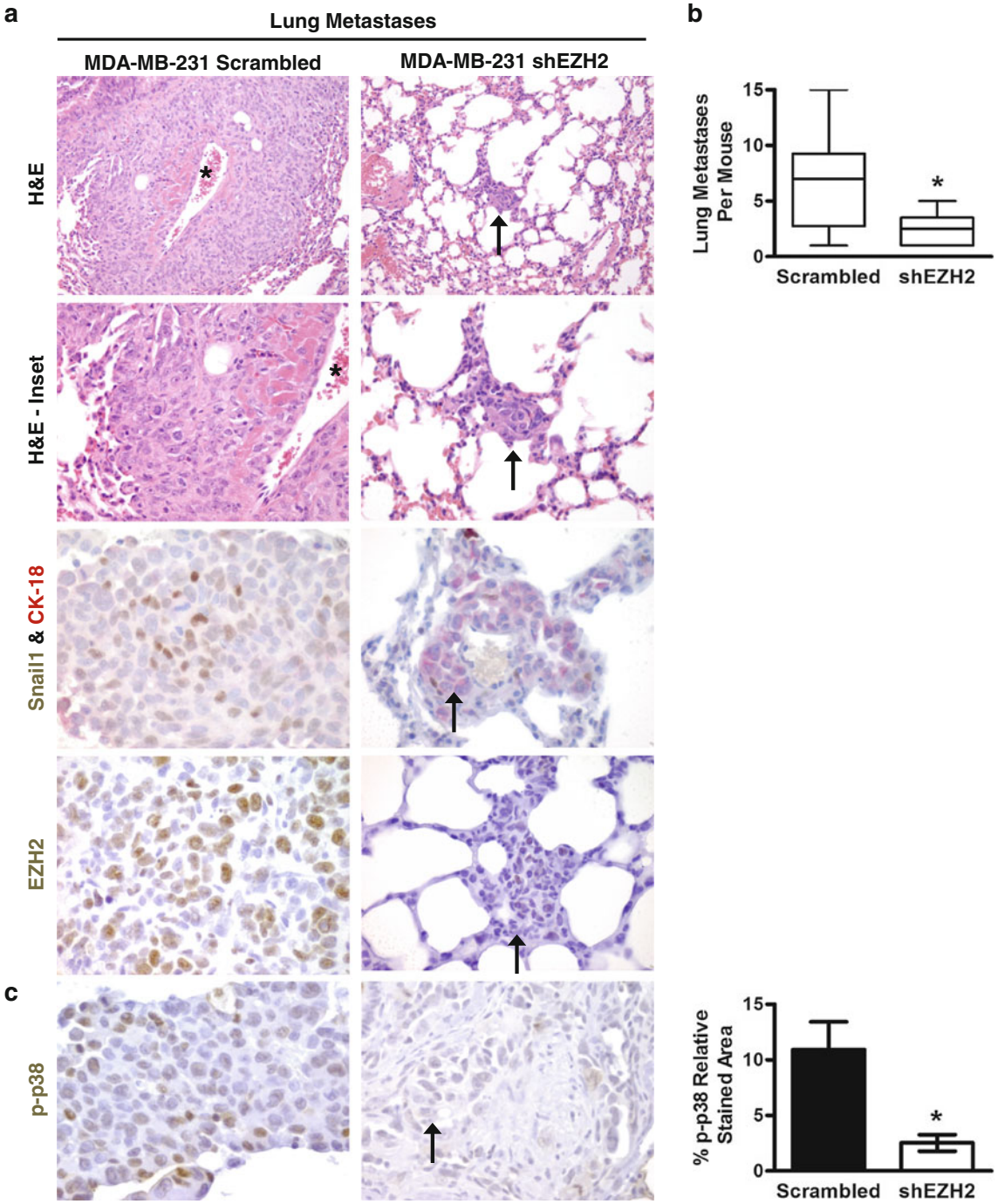
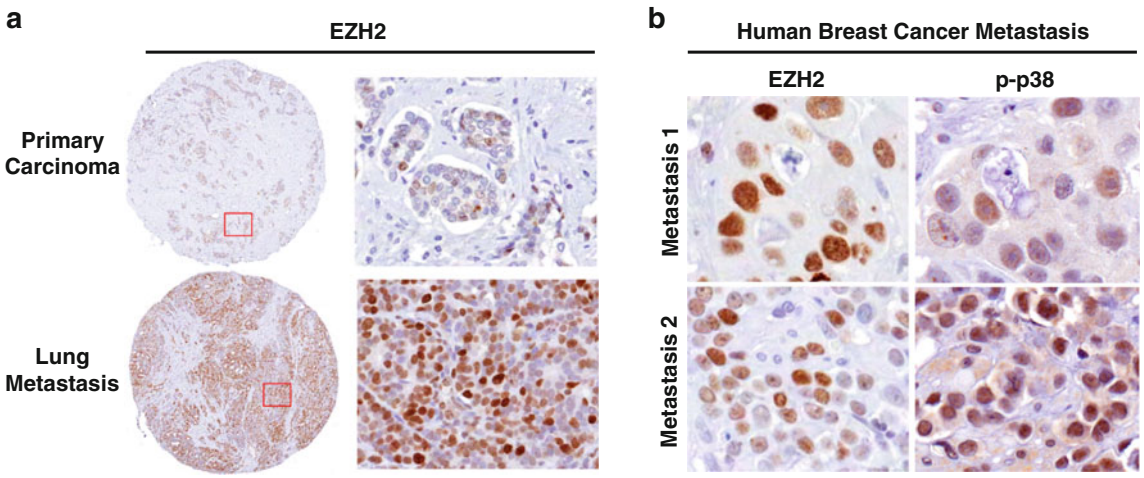


Fig. 4 EZH2 knockdown in MDA-MB-231 cells is sufficient to reduce distant metastasis. **a** Representative photomicrographs of mouse lung metastases of MDA-MB-231 scrambled shRNA control or EZH2-targeted shRNA (shEZH2) cells. EZH2 KD changed the tumor morphology from poorly circumscribed and highly invasive areas toward small, circumscribed foci. The *asterisk* shows a vessel encased by metastatic carcinoma. The *arrows* indicate metastases formed by MDA-MB-231 shEZH2 cells. Double immunostain with anti-CK-18 and anti-Snail1 antibodies show that shEZH2 metastases exhibit upregulation of CK-18 and decreased expression of Snail1 in the nuclei of cancer cells compared to controls (H&E: ×200 magnification; H&E-*inset*: ×400 magnification; CK-18&Snail1, EZH2: ×600 magnification]. **b** EZH2 KD significantly reduced the number of lung metastases per mouse. The shows that MDA-MB-231 shEZH2 cells formed significantly fewer lung metastases compared to controls. *Whiskers* indicate the minimum and maximum number of lung metastases per mouse for each condition (Student's *t* test, $*p < 0.05$). **c** *Left*, photomicrographs of lung metastases of MDA-MB-231 control and shEZH2 cells exhibit decreased p-p38 protein (×600 magnification). *Right*, *bar graph* shows p-p38 protein expression ± SEM in shEZH2 and control lung metastases quantified using FRIDA software (Student's *t* test, $*p = 0.01$)

mesenchymal states [29, 30]. EMT describes the reversible and dynamic process in which epithelial cells, characterized as organized and polarized, undergo a change into mesenchymal-like cells, with alterations in cell polarization and intercellular junctions. The process of epithelial cancer cells acquiring attributes of mesenchymal-like cells has been shown to promote a motile state and to induce the development of metastasis [29]. One of the hallmarks of EMT is the reduction of normal expression of the cell–cell junction protein E-cadherin [30]. We and other investigators have reported that EZH2 overexpression induces invasion in non-tumorigenic breast cells, and decreases the expression of E-cadherin [14, 23]. However, whether EZH2 can influence EMT and motility of breast cancer cells has not been previously considered. In this study, we show that EZH2 downregulation is sufficient to reprogram the phenotype of breast cancer cells from spindle towards epithelial. EZH2 knockdown led to upregulation of the epithelial proteins E-cadherin and Cytokeratin-18, downregulation of the mesenchymal protein Vimentin and the EMT transcription factor Snail1, and resulted in decreased motility of breast cancer cells.

The p38 MAPK has been established as a regulator of transitions between epithelial and mesenchymal states as well as cancer cell migration [20, 21, 27]. Activated p-p38 regulates transcription factors responsible for E-cadherin repression including Snail1, Slug (Snail2), and Twist inducing a mesenchymal-like phenotype [31–34]. During TGF-β-induced EMT, p38 activation increases breast cancer lung metastasis [35]. p38α activity is required for the invasive capability of breast, pancreatic, hepatocellular, and head and neck squamous carcinoma cell lines, in part through regulation of matrix metalloproteinases implicated in extracellular remodeling and degradation [27, 36–44]. Also, p38δ has been proposed to regulate the invasion of squamous cell carcinoma, while p38γ has been associated with Ras-induced invasion [37, 45]. Recently, down-regulation of p38γ markedly decreased the cell motility of breast cancer cells in vitro [21]. Despite the important role of p38 in motility, invasion, and metastasis of human



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	EZH2 low	EZH2 high	Total	p-p38 low	p-p38 high	Total
Primary Carcinoma	7 (43.8%)	9 (56.3%)	16	11 (73.3%)	4 (26.7%)	15
Metastasis	0	16 (100%)	16	6 (37.5%)	10 (62.5%)	16
	Fisher's Exact Test, p<0.007			Fisher's Exact Test, p<0.05		

Fig. 5 EZH2 and p-p38 are significantly upregulated in human breast cancer metastases when compared to matched primary tumors from the same patient. **a** Representative images of matched primary human breast carcinomas and metastases ($n = 16$ patients) immunostained for EZH2 (×100 magnification, *inset*: ×400 magnification). EZH2 is upregulated in the metastasis compared to the primary tumor. **b** Representative images of two metastases showing concordant high EZH2 and p-p38 expression (×600 magnification). **c** The *table* shows the distribution of EZH2 and p-p38 protein expression in the 16 primary breast carcinomas and matched metastases; 62.5 % of metastases exhibited high expression of both EZH2 and p-p38

cancer, the mechanisms regulating its activation are still being defined. Through a combination of knockdown and overexpression strategies, we found that EZH2 is a novel regulator of p-p38 protein levels and signaling pathway in non-tumorigenic breast cells and breast cancer cell lines.

The mechanisms implicated in the oncogenic role of EZH2 need further investigation. EZH2 has been considered largely a transcriptional repressor of tumor suppressor genes as part of PRC2, but recent evidence supports contextual, activating functions of EZH2 [7–10, 46]. Here, we demonstrate that EZH2 regulates p-p38 via a non-transcriptional mechanism. EZH2 had no effect on the mRNA levels of p38 isoforms using quantitative RT-PCR. Unexpectedly, in breast cancer cells, we found that endogenous EZH2, EED, and SUZ12 proteins bind to p-p38 protein, and that downregulation of EZH2 abrogates the binding of EZH2 and p-p38. Our in vitro methylation assay results suggest that PRC2 may methylate p-p38, and paves the way for future studies. Our data lead to the novel hypothesis that EZH2 in association with other PRC2 members may influence p-p38 activity, which is under investigation in our laboratory.

Our group has previously reported that high EZH2 protein expression is associated with the development of metastasis in breast cancer and worse clinical outcome [14]. Data presented here show for the first time that EZH2 knockdown reduces the number of distant breast cancer metastases in vivo. EZH2 knockdown in highly aggressive MDA-MB-231 cells decreased the metastatic burden and reduced the invasiveness of breast cancer cells at the metastatic site, as well as the expression of p-p38. In paired human samples of primary and metastatic carcinomas, EZH2 was significantly overexpressed in the metastases. Furthermore, co-expression of EZH2 and p-p38 were detected in 63 % of the metastatic carcinomas.

In conclusion, our results demonstrate a previously unknown mechanism of EZH2 function in breast cancer metastasis. We have shown that EZH2 inhibition in breast cancer cell lines leads to a phenotypic change from mesenchymal to epithelial, with reduced motility, invasion, and metastasis. We uncover a previously unknown molecular mechanism by which EZH2 binds to p-p38 and regulates the activation of the p38 signaling pathway. From a clinical perspective, the role of EZH2 in p38 signaling is of particular interest as activation of this pathway can be detectable and targetable in tumors to reduce breast cancer metastasis.

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Conflict of interest The authors declare that they have no conflict of interest.

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EZH2 inhibition decreases p38 signaling and suppresses breast cancer motility and metastasis

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Supplementary Methods

Real-time RT-PCR

Quantitative real-time reverse transcription PCR (RT-PCR) amplifications were carried out with 1 μg of total RNA isolated from the indicated breast cells and conditions. Reactions were performed in triplicate with Qiagen primers and SYBR Green Master Mix (Applied Biosystems, #4309155). All primers were purchased from Qiagen: GAPDH #PPH00150E-200, MAPK1 (Total p38) #PPH00715B-200, MAPK14 (p38α) #PPH00750B-200, MAPK11 (p38β) #PPH01778B-200, MAPK12 (p38γ) #PPH01779A-200, and MAPK13 (p38δ) #PPH00188B-200.

In vitro methylation

The *in vitro* methylation assay was completed as previously described [1]. Briefly, 1 μg recombinant p38α-GST (BPS Bioscience, #40070) was incubated with 5 μl methylation buffer (20 mM Tris-HCl [pH 7.8], 5mM DTT, 0.5mM EDTA, 10% glycerol) in the presence of 10 μM H^3-S-Adenosylmethionine, with or without the presence of 2 μg purified PRC2 complex (total protein amount), at 30°C for 1 hr. Samples were then separated via SDS-PAGE, the gel was coomassie-stained to visualize proteins, and methylation was detected by autoradiograph at 21 days.

Spontaneous metastasis assay

Briefly, MDA-MB-231 cells expressing shEZH2 or scrambled control were orthotopically injected into the mammary fat pad at a concentration of 2×10^6 cells in 20 mice (n=10 per group). Tumor

size was measured twice a week until tumors reached 2 cm^3, at which time mice were sacrificed and lung tissues collected. Tissues were formalin-fixed and paraffin-embedded for hematoxylin and eosin staining and immunohistochemical staining with anti-EZH2 (Cell Signaling, #5246), anti-Cytokeratin-18 (abcam, #32118, 1:100), anti-Snail1 (Cell Signaling, #3895, 1:800), anti-Ki67 (Fisher, #RM-9106, 1:2000), or anti-ACTIVE®-p38 MAPK (Promega, #V1211, 1:325). Image analysis and quantification of only metastatic cells to determine the percentage of relative stained area was completed using FRIDA (FRamework for Image Dataset Analysis), a custom open source image analysis software package for the analysis of RGB color image datasets [2]. Additional information can be found at http://bui3.win.ad.jhu.edu/frida/. All procedures involving mice were approved by the University Committee on Use and Care of Animals at the University of Michigan and conform to their relevant regulatory standards.

Supplementary Methods References

1. Wu L, Zee BM, Wang Y, Garcia BA, Dou Y (2011) The RING finger protein MSL2 in the MOF complex is an E3 ubiquitin ligase for H2B K34 and is involved in crosstalk with H3 K4 and K79 methylation. Mol Cell 43 (1):132-144. doi:S1097-2765(11)00382-0 [pii] 10.1016/j.molcel.2011.05.015

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Supplementary Figure Legends

Supplementary Figure 1 EZH2 knockdown induces a mesenchymal-to-epithelial transition and decreases invasion in breast cancer cells. **(a)** Immunoblots of SUM149 and MDA-MB-231 breast cancer cells show that downregulation of EZH2 protein with 1μM DZNeP treatment leads to a protein expression profile indicative of epithelial differentiation compared to untreated control cells. E-cadherin and Cytokeratin-18 [CK-18] represent epithelial marker proteins, and Vimentin and Snail1 represent mesenchymal marker proteins. **(b)** Representative phase contrast images show that EZH2 knockdown with DZNeP in SUM149 and MDA-MB-231 cells leads to a morphological change from mesenchymal-like to epithelial when compared to controls [200X magnification]. **(c)** EZH2 inhibition with 1 μM DZNeP reduces invasion of SUM149 and MDA-MB-231 cells compared to untreated controls using a reconstituted Boyden basement membrane invasion chamber assay. Left, representative fields of invaded and stained Boyden chambers are shown [200X magnification]; right, invasion was quantified using colorimetry with absorbance at 560 nm ±SD [Student's t-test, *p=0.002, **p=0.01].

Supplementary Figure 2 EZH2 regulates the activation of total and isoform specific protein levels of p38. (a) Immunoblots of SUM149 and MDA-MB-231 breast cancer cells show that EZH2 knockdown with DZNeP treatment decreases the levels of p-p38 and its activity as demonstrated by the phosphorylation of downstream signaling targets, MK2 and HSP27. **(b)** Immunoblots of MCF7 breast cancer cells and nontumorigenic MCF10A breast epithelial cells show that p-p38 levels are increased with adenoviral myc-tagged EZH2 over-expression when compared to controls.

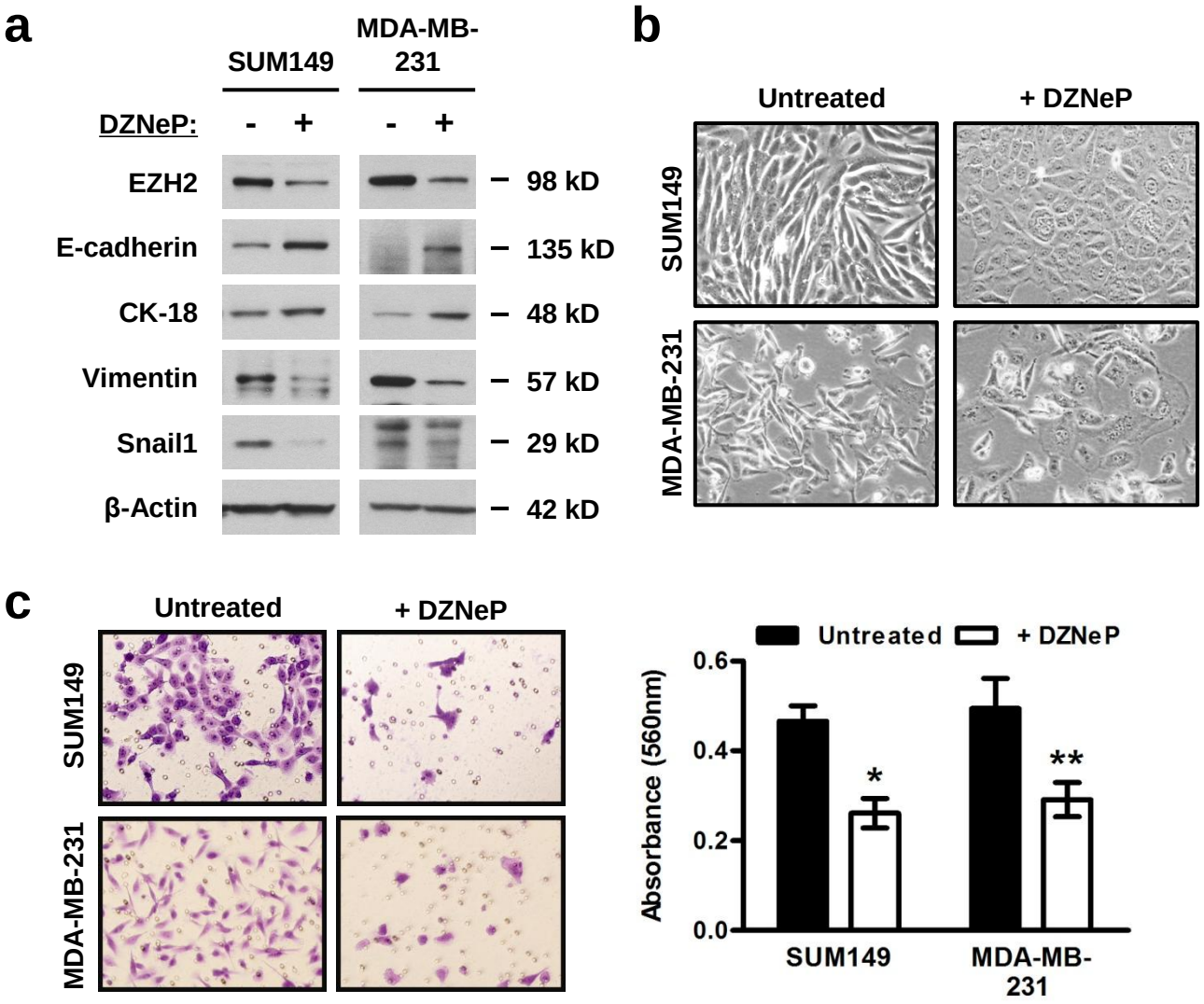
Supplementary Figure 3 EZH2 regulates the activation of the p38 MAPK signaling pathway and the PRC2 complex can methylate p38α *in vitro*. (a) Activated, phosphorylated levels of all four p38 isoforms, but not total isoform protein levels, are decreased in MDA-MB-231 shEZH2 cells when compared to scrambled shRNA control cells. Total p-p38 was immunoprecipitated from whole cell

extracts followed by Western blot analysis for the four individual isoforms. **(b)** Quantitative real-time RT-PCR reveals that EZH2 KD in SUM149 and MDA-MB-231 breast cancer cells or transient adenoviral overexpression in nontumorigenic MCF10A breast cells has no significant effect on the mRNA levels of total p38 or of any of the four p38 isoforms when compared to controls. mRNA expression is shown relative to GAPDH mRNA levels ± SD. **(c)** *In vitro* methylation assay reveals that addition of the PRC2 complex leads to the methylation of GST-p38α.

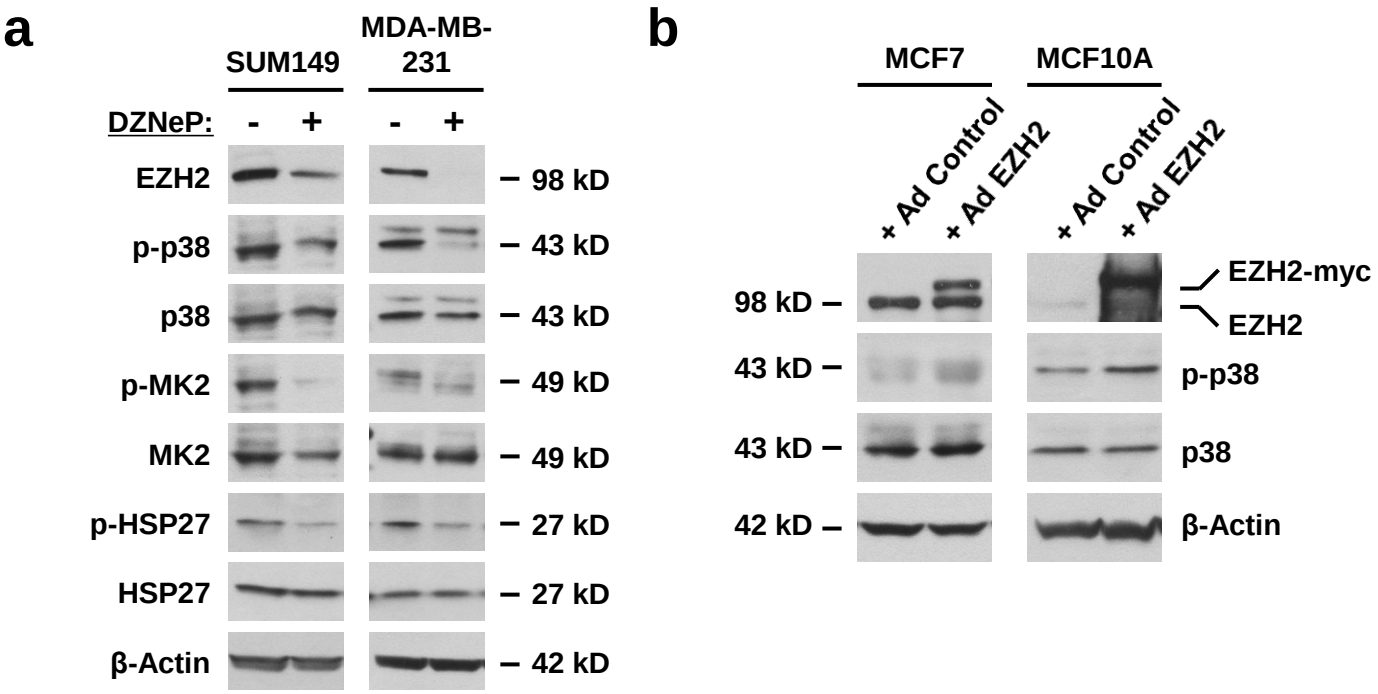
Supplementary Figure 4 MDA-MB-231 shEZH2 primary xenografts show higher CK-18 and lower Snail1 expression, and MDA-MB-231 shEZH2 lung metastases have significantly reduced expression of the Ki67 proliferative cell marker when compared to MDA-MB-231 control lung metastases. (a) Representative photomicrographs of MDA-MB-231 scrambled control and shEZH2 primary xenografts with immunostaining for the epithelial marker CK-18 (red) and the mesenchymal marker Snail1 (brown) [400X magnification]. Arrow indicates the invasive edge of the tumor, on right, into the surrounding stroma, on left. **(b)** Left, representative photomicrographs of MDA-MB-231 control and shEZH2 lung metastases with Ki67 immunostaining [400X magnification]. Dotted line indicates boundaries of the shEZH2 lung metastasis. Right, bar graph shows Ki67 protein expression ± SEM in shEZH2 and control lung metastases quantified using FRIDA software [Student's t-test, *p=0.03].

Supplementary Table 1 Complete clinical and pathological information, including EZH2 and p-p38 protein expression, for the tumor microarrays containing 16 human primary breast carcinomas with matched metastases.

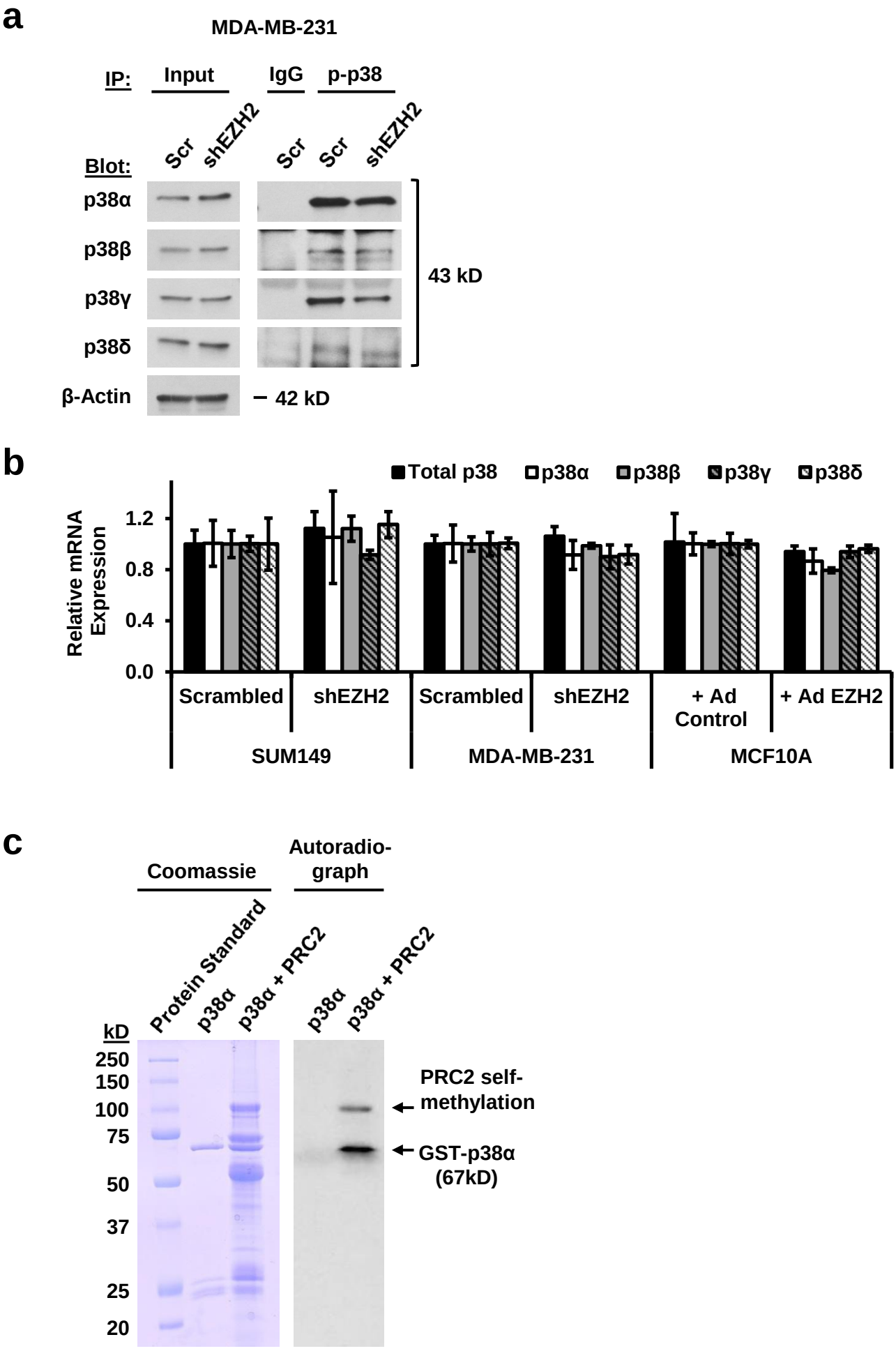
Supplementary Figure 1



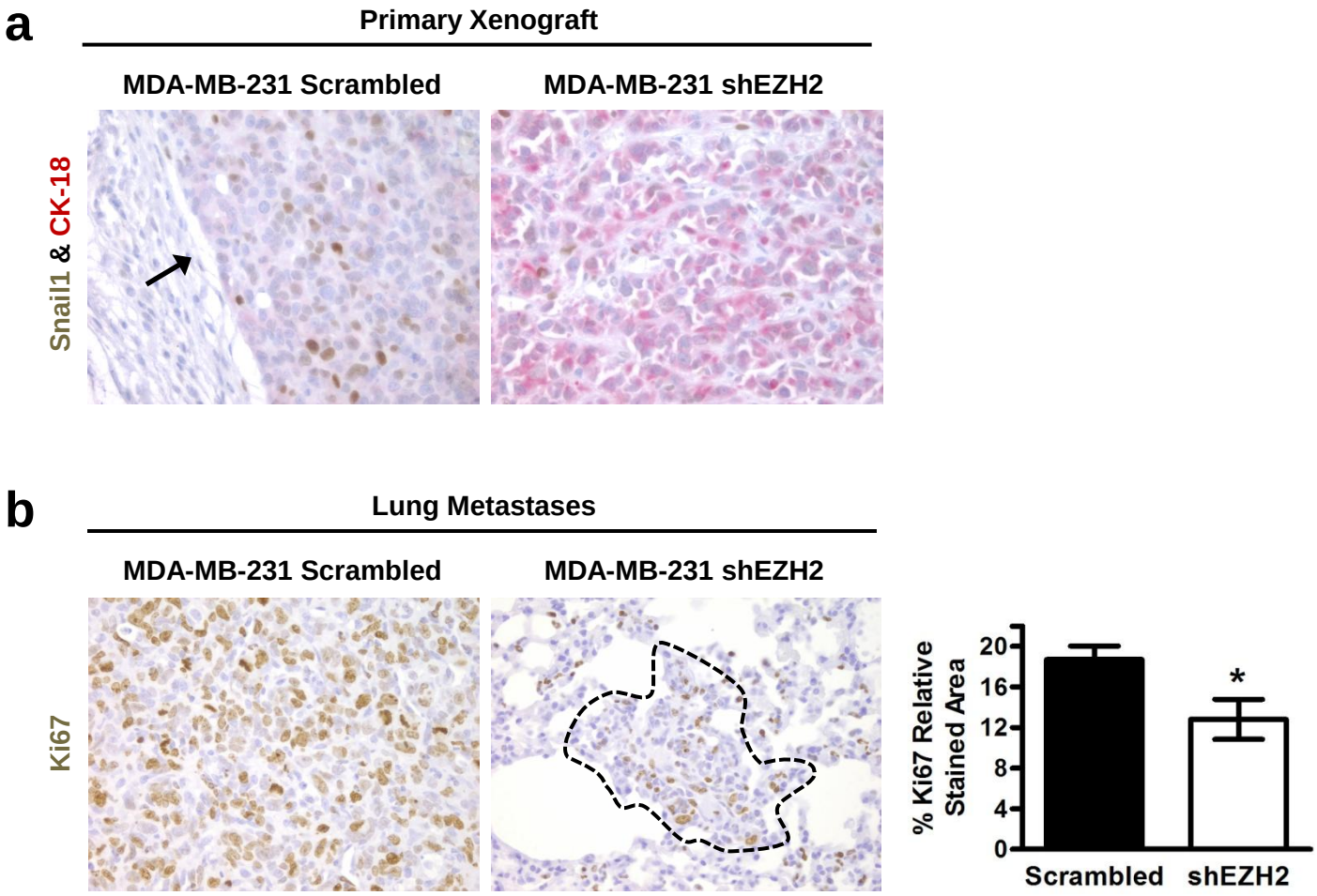
Supplementary Figure 2



Supplementary Figure 3



Supplementary Figure 4



Supplementary Table 1.

							Primary Carcinoma					EZH2 (ow or high)		p-p38 (low or high)	
Case	Age at Diagnosis (years)	Pathologic Stage at Diagnosis	Primary Tumor Size (cm)	Nodal Status at Diagnosis	Tumor Type & Grade	Subtype	ER Status	PR Status	Her2 Status	Interval to Metastasis (years)	Metastasis Location	Primary Carcinoma	Metastasis	Primary Carcinoma	Metastasis
1	33	T3N1M1	6	5/24 positive	IDC, Grade 3	Her2	-	-	3+	At diagnosis	Lung	H	H	L	L
2	50	T2N1	2.3	1/19 positive	IDC, Grade 3	Luminal	+	+	0	6	Lung	L	H	L	L
3	34	T2N2M1	4.6	4/9 positive	IDC, Grade 3	TNC	-	-	0	At diagnosis	Brain	H	H	N/A	L
4	38	T1cN0	1.9	0	ILC, Grade 2	Luminal	+	+	1+	3	Ovary	L	H	L	H
5	45	T1aN1	< 0.5	1/3 positive	IDC, Grade 3	Luminal	+	+	0	7	Lung	L	H	L	L
6	36	yT1cN0	1.8	0	IDC, Grade 2	Her2	-	-	3+	4	Brain	L	H	L	H
7	39	T2N0	3	0	IDC, Grade 3	TNC	-	-	0	4	Brain	H	H	L	H
8	53	TXNX	radiologic size 7 cm	1/31 positive	IDC, Grade 3	TNC	-	-	0	1	Brain	L	H	L	L
9	38	T1cN0	1.4	0	IDC, Grade 3	TNC	-	-	0	5	Brain	H	H	H	L
10	53	T2N2	3.5	7/20 positive	IDC, Grade 3	Luminal	+	+	0	5	Brain	H	H	H	H
11	58	T2N0	3	0	ILC, Grade 2	Luminal	+	-	0	7	GI-Bowel	L	H	L	H
12	56	TXNXM1	diffuse radiologic abnormality	X	ILC, Grade 2	Luminal	+	+	0	At diagnosis	GI-Bowel	L	H	L	H
13	44	T2N0	2.6	0	IDC, Grade 3	TNC	-	-	0	1	Lung	H	H	L	H
14	33	yT2N1mi	3.7	micromet	IDC, Grade 2	Luminal	+	+	0	2	GI-Pancreas	H	H	H	H
15	57	T1N0	1.8	0	IDC, Grade 3	TNC	-	-	0	6	Lung	H	H	L	H
16	36	T1N0	multiple foci <2 cm	0	IDC, Grade 3	Luminal	+	+	0	2	Brain	H	H	H	H